

Fitness, persistence, and responsiveness of a genetically engineered strain of *Trichoderma virens* in soil mesocosms

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Abstract

The genetic stability and ecological persistence of a strain of the filamentous fungus, *Trichoderma virens*, genetically modified (GM) with a hygromycin resistance gene and a gene encoding an organophosphohydrolase was evaluated over 243 days in soil mesocosms. The GM populations declined over time, a trend similar to the wild-type, parental strain (WT), but was still present at the end of the incubation. Similar population estimates were obtained by plating serial dilutions on selective media or by quantitative polymerase chain reaction (QC-PCR) after 243 days' incubation. However, the absolute population size differed between the two methods by more than a log order of magnitude with QC-PCR being more sensitive. The presence of the WT strain did not adversely affect the persistence of the GM strain. The GM strain stably maintained the transgene and still expressed antibiotic resistance and the ability to degrade organophosphate xenobiotics. Even after the long-term incubation the GM and WT strains rapidly and efficiently colonized freshly added substrates, indicating a high level of responsiveness.

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1. Introduction

The public debate over release of genetically modified organisms (GMOs) has increased in intensity. Much of the recent debate has focused on the safety of genetically modified (GM) agronomic crops for human consumption (Braun, 2002; Hellmich et al.,

2001; Kuiper et al., 2002; Miles and Frewer, 2001; Sears et al., 2001; Wal, 2002), regulatory aspects of GM crops (Gaugitsch, 2002; Owen, 2000; Schilter and Constable, 2002), the impact of GMO derived pesticides on ecosystems (Stanley-Horn et al., 2001; Hails, 2000; Jesse and Obrycki, 2000; Johnson and Gould, 1992), and the possibility of escape and entrenchment of GMOs or foreign genes outside their intended environments (Chadoeuf et al., 1998; Faure et al., 2002; Kluepfel et al., 1991; Kuvshinov et al., 2001). These last two concerns are frequently

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expressed over large scale planting of crops such as *Bt*-producing maize (Stanley-Horn et al., 2001; Hellmich et al., 2001; Sears et al., 2001; Wal, 2002) or glyphosate-resistant canola (Rieger et al., 2002; Rieger et al., 1999). Escape and entrenchment are also potential outcomes resulting from the deployment of genetically engineered microorganisms (GEMs).

Early reviews on the field release of GEMs noted the extensive regulatory burden to obtain permission from the Environmental Protection Agency (EPA), the US Department of Agriculture, Animal and Plant Health Inspection Service (USDA APHIS) and the expensive containment measures and tedious monitoring for spread of the GEM (Skirvin et al., 2000; Wilson and Lindow, 1993). The current regulatory climate is more relaxed but restrictions on field deployment on GEMs remain a significant obstacle. The continued, albeit reduced, regulatory burden is a response to the perceived risks and uncertainty regarding the environmental fate of GEMs.

The surest means of removing the uncertainty is through well-monitored field releases. Unfortunately, the potential negative consequences from a field release are catastrophic. Thus, pragmatism dictates that information is first gathered from more easily controlled, laboratory-contained experiments. These experiments are more easily contained, easier to monitor, and can be performed on limited budgets. We report here on one such laboratory-contained experiment with a genetically modified strain of the saprophytic fungus, *Trichoderma virens* (Miller, Giddens and Foster) Arx (Anamorph). This fungus efficiently colonizes the rhizosphere of numerous plants including agronomic crops, ornamentals and turf species (Harman, 1990; Harman et al., 1996; Hjeljord and Tronsmo, 1998; Papavizas, 1985) and is tolerant to a very broad range of substrates including many agrochemicals (Howell, 1996; Park et al., 1992b). Because of these traits, *T. virens* is an attractive model for creating an improved agent for bioremediation. A well-studied strain of *T. virens* (Park et al., 1992a,b) was heterologously transformed with an antibiotic resistance gene and a gene for an organophosphate hydrolase (Dave et al., 1994). An image analysis and modeling approach described the growth and branching patterns of the GM and wild-type (WT) strain (Cross and Kenerley, 2004). These studies have detected significant, quantitative,

differences between the WT and the GM strains, but failed to detect qualitative, pleiotrophic effects of the transgenes; both strains are highly rhizosphere competent and have similar fractal growth (Classen et al., 1996). While *T. virens* has a long history of safe deployment, the addition of foreign genes forces a reevaluation of the ecology of the organism to better predict the environmental fate of the transgene. We tested one strain, genetically engineered with two foreign genes, and its wild-type progenitor strain in soil mesocosms to evaluate the WT and GM strain's relative fitness, persistence and responsiveness to substrate introduction.

2. Materials and methods

2.1. *Trichoderma virens* strains and media

A wild type strain, Gv29-8 was isolated from agricultural soil near College Station, TX (Park et al., 1992a,b), maintained on potato dextrose agar (PDA; Difco Laboratories). The genetically engineered strain, GvT6 was constructed by transformation and heterologous integration of the plasmid pCL1, which included the hygromycin resistance gene, *hygB* and a gene encoding for organophosphate hydrolase (OPH), *opd* (Dave et al., 1994). This hydrolase has a broad spectrum of activity against numerous phosphotriesters such as methyl and ethyl parathion, dursban, diazion, paraoxon, and the fluorophosphate neurotoxins sarin and soman (Dumas et al., 1989). GvT6 was selected based on high expression levels of OPH and stable hygromycin resistance (Dave et al., 1994). For chlamydospore production, conidia were added to molasses medium (Papavizas et al., 1984) and harvested after 14 days. Soil was obtained from a cotton field near College Station, TX, and was chemically and texturally characterized (Baek and Kenerley, 1998).

2.2. Soil infestation and incubation

2.2.1. Responsiveness of *T. virens* to dried cotton roots

A preliminary experiment evaluated the responsiveness of *T. virens* to addition of a natural carbon source. Biomass from strain WT was collected from

the molasses medium by vacuum filtration and added to soil that had been passed through a 2-mm sieve. Soil moisture was adjusted to -0.05 MPa (13% moisture) and to a density of 6.8×10^3 CFU/g. Dried, milled cotton roots were added to a concentration of 0, 0.5, 1, 1.5 or 2% of the soil weight. The population density of *T. virens* was determined after 18 days' incubation.

2.2.2. Long-term survival and responsiveness

Biomass from both strains was collected from the molasses medium. Soil was prepared that contained either the WT strain or the GM strain or both strains together (in a ratio of 1:1). The soil was mixed thoroughly and sampled to determine the number of colony forming units (CFU) per gram of soil. After 3 days, additional soil was added to adjust the population density to 1 million CFU/g of soil. Water was added to adjust the soil water potential to -0.05 MPa. Some treatments also received a supplement of 0.5% dried, milled cotton roots. Infested soil was placed in 4 cm \times 30 cm polyvinylchloride tubes, which were placed in growth chambers (Environmental Growth Chambers) where they were maintained at one of three temperature regimes (Fig. 1). The temperatures were selected to reflect a constant incubation temperature (Temp I) and the temperature

fluctuations expected at 2 and 5 cm depth (Temp II and Temp III, respectively) in Texas cotton fields. All 18 treatments [strain (WT, GM or mixture), amendment (with and without supplemental cotton roots), and temperature (one of three regimes)] were repeated three times for a total of 54 replicates.

2.3. Microbiological estimation of *T. virens* population size

Over a 243 days' incubation period, the soil mesocosms were sampled 13 times by emptying the mesocosms and mixing the soil on each sampling occasion. Population levels in soil were determined by diluting soil samples in water and plating on selective medium, GVSM (39 g potato dextrose agar, 2.5 g Triton X 100, 200 μ g benomyl, 50 mg rifampicin, 500 mg sodium propionate, 50 mg streptomycin sulfate, 50 mg rose bengal and 40 mg of a crude preparation of gliotoxin) or GVSM with the addition of hygromycin (liquid equivalent of 100 mg/L) (Calbiochem). These plates were monitored for 2–5 days to determine the phenotype and number of *T. virens* CFU/g of soil. After the mesocosms were mixed, samples were taken and the moisture content determined by oven drying the soil. Water was added as needed to adjust the water potential to -0.05 MPa.

2.4. Molecular estimation of *T. virens* population size

Samples taken after 243 days' incubation were also analyzed by quantitative polymerase chain reaction (QC-PCR) (Baek and Kenerley, 1998). Briefly, 0.5 g of soil was mixed with 500 μ L of extraction buffer (100 mM Tris-HCL, 100 mM EDTA [pH 8.0] 100 mM sodium phosphate, 1.5 mM NaCl, 1% hexadecyltrimethylammoniumbromide, 2% sodium dodecyl sulfate). The mixture was incubated at 65 $^{\circ}$ C for 2 h and centrifuged. The pellet was re-extracted with 250 μ L of buffer and then mixed with 1 g zirconia/silica beads and 250 μ L of buffer for 3 min with a bead-beater. The supernatants were combined, extracted with phenol/chloroform and nucleic acids were precipitated with isopropyl alcohol. Nucleic acids were further purified with Wizard DNA mini-columns (Promega). These DNA extracts competed with a PCR "mimic" for binding to oligonucleotide primers with homology to either the OPD

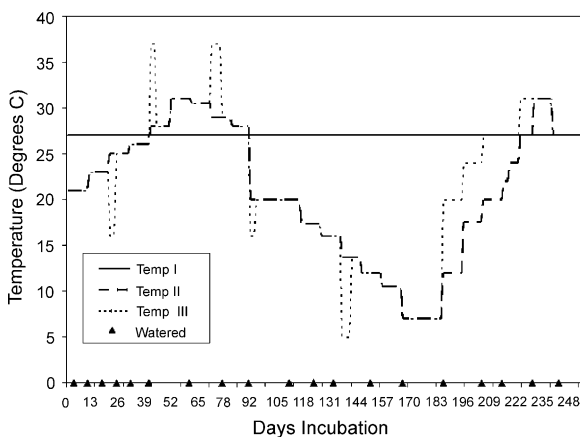


Fig. 1. Incubation conditions: the three lines represent the temperature of the soil incubators. One soil incubator was maintained at a constant 27 $^{\circ}$ C (Temp I) while the other two temperature regimes reflected the soil temperature fluctuations typical of Texas cotton fields at 2 and 5 cm depth (Temp II and Temp III, respectively). Arrows mark points at which the water content was measured and adjusted.

gene for the GM strain or a peptide synthetase gene for both strains. Polymerase chain reagents included 20 mM Tris-HCL, 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM deoxyribonucleotide triphosphate and 12.5 pmol of the primers 5'-TCGATCGGCACAGGCAG-ATCGATGGCTCCATCAAGGTCGG-3' and 5'-TG-ACGCCCCGAAGGTCGGTGTGTTGGAGGCATCG-ACCTTGG-3' for enumeration of the GM strain or 5'-TCGATGGCTCCATCAAGGTCGG-3' and 5'-TTTGGAGGCATCGACCTTGG-3' for enumeration of the WT strain (Baek and Kenerley, 1998).

2.5. Characterization of the stability of genetically engineered strain

Previously we had observed that isolates of GvT6 could be recovered from infested soil that were resistant to hygromycin, but no longer produced OPH. We were interested if this genetic instability or loss of OPH expression would appear among the isolates recovered during this experiment. To assess the stability of the foreign genes (*opd* and *hygB*) in recovered isolates, 100 hygromycin-resistant colonies were assayed for production of OPH at each sampling time. Conidia ($\sim 1 \times 10^6$ /mL) from each colony were inoculated into 12 mL of Vogel's minimal medium (Vogel, 1956) supplemented with sucrose (1.5%, w/v) (VMS) buffered with 0.1 M HEPES to pH 7.6 and shaken at room temperature for 5 days. Culture filtrate was tested for enzyme activity in a 96-well microtiter plate. Culture filtrate (270 μ L) from colonies grown in VMS was added to each well followed by 30 μ L of 0.794 mM paraoxon in 50 mM CHES pH 9 (Cross and Kenerley, 2004). Activity was measured using a microtiter plate spectrophotometer, ABS 400. Strains GvT6 and TV 29-8 were used as positive and negative controls, respectively.

2.6. Characterization of the responsiveness of *T. virens* populations

Five times during this experiment, 2 mm sections of small, washed cotton roots were placed in nylon mesh envelopes and incubated in the soil mesocosms that contained the mixtures of both populations. After 2 days' incubation the root sections from each mesocosm were removed from the envelopes, placed in sterile water (100 mL) shaken for 1 h, transferred to

a second flask of sterile water, shaken for an additional 30 min, removed and blotted dry on sterile filter paper. The washed roots were plated directly on GVSM or GVSM-hygromycin or were homogenized in water, diluted in water and aliquots spread on selective media.

2.7. Modeling of the survival of *T. virens*

The survival of the *T. virens* populations were plotted against time for each treatment and visually inspected for trends. These data were fitted to a modified logistic growth equation of the form:

$$\frac{dN}{dt} = r_{\gamma} N (1 - N/K_{\gamma})$$

where $K_{\gamma}(t) = \xi(E_0 + \gamma t - \mu/\lambda) - \beta(t)$. γ is the resource replenishment rate, ξ the dimensionless factor that relates nutrient level to population density, E_0 the initial total vital bioenergy per unit dry soil, μ the innate mortality rate, λ the fecundity rate per unit accessible resources and $r_{\gamma}(t)$ is the K_{γ} -dependent intrinsic growth rate (Wu et al., 2000). This equation was recently derived to model the long-term survival of *T. virens* in soil. Survival curves were compared statistically using the repeated measures AVOVA (SAS Cary, NC).

3. Results

3.1. Infestation of soil

Both strains grew rapidly in the molasses medium, but differed from the morphology described by others in this medium (Papavizas et al., 1984). Both strains tested here consistently produced aggregates of chlamydospores that were still attached to one another by hyphae (Fig. 2).

3.2. Growth response to cotton roots

The WT populations significantly increased with the addition of all amounts of dried, milled cotton roots to the soil as compared to the control. After 18 days' incubation in soil without additional substrate, the WT population was 7×10^5 CFU/g soil. With cotton roots added at a rate of 10 g/kg, the population

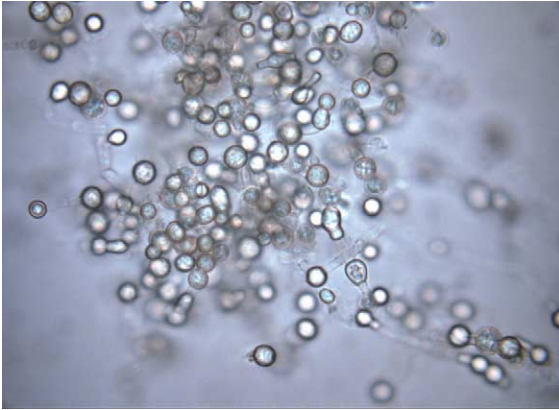


Fig. 2. Micrograph of an aggregate of chlamydospores produced by *T. virens* in molasses medium after 14 days' of incubation.

was 1.7×10^6 CFU/g soil. The addition of cotton roots at rates of 15 or 20 g/kg did not result in a significant increase in the population of WT as compared to the 10 g/kg rate. The populations were similar for these highest rates of cotton roots.

3.3. Long-term survival of *T. virens* in soil

The survival curves of all populations, as determined by selective plating are presented in Figs. 3 and 4. Generally, there was a slow decline in population size over time. While all populations, in all treatments asymptotically approached the limit of detection by selective plating, the mean population never reached zero in any treatment. Many treatments including the WT strain had an initial increase in the population size. Repeated measures statistical analysis indicated

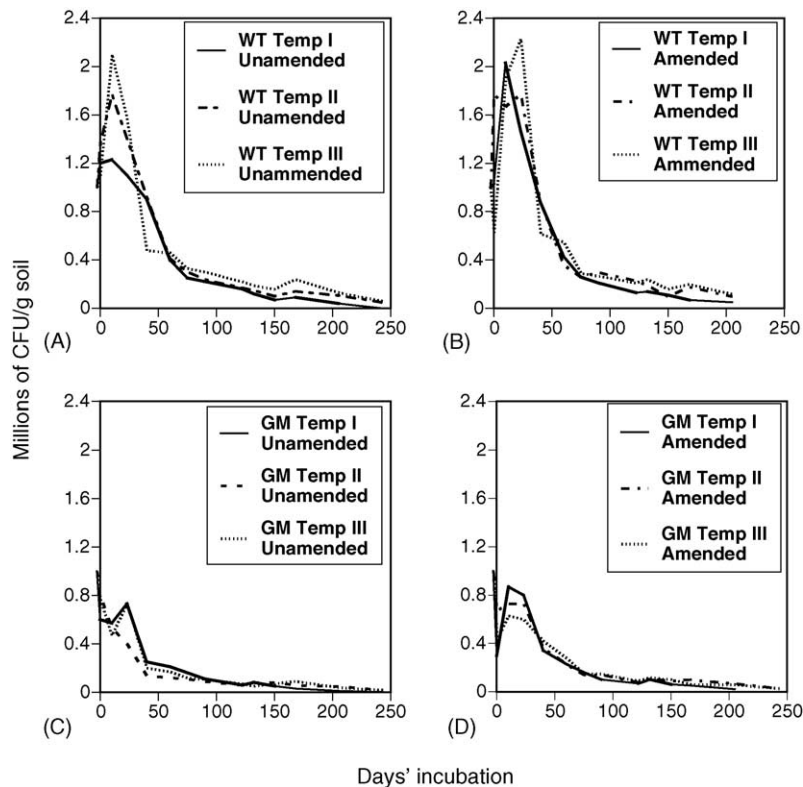


Fig. 3. Population dynamics of *T. virens* monocultures in soil mesocosms. Wild type (WT) strain dynamics are presented in panel (A and B); genetically modified (GM) strain dynamics are presented in (C and D). Population dynamics in soil mesocosms are presented in panels (A and C); dynamics in soil mesocosms amended with dried, milled cotton roots are presented in (B and D). The temperatures are as in Fig. 1 (Temp I, constant 27°; Temp II and Temp III seasonal fluctuations in a Texas cotton files at 2 and 5 cm depth, respectively).

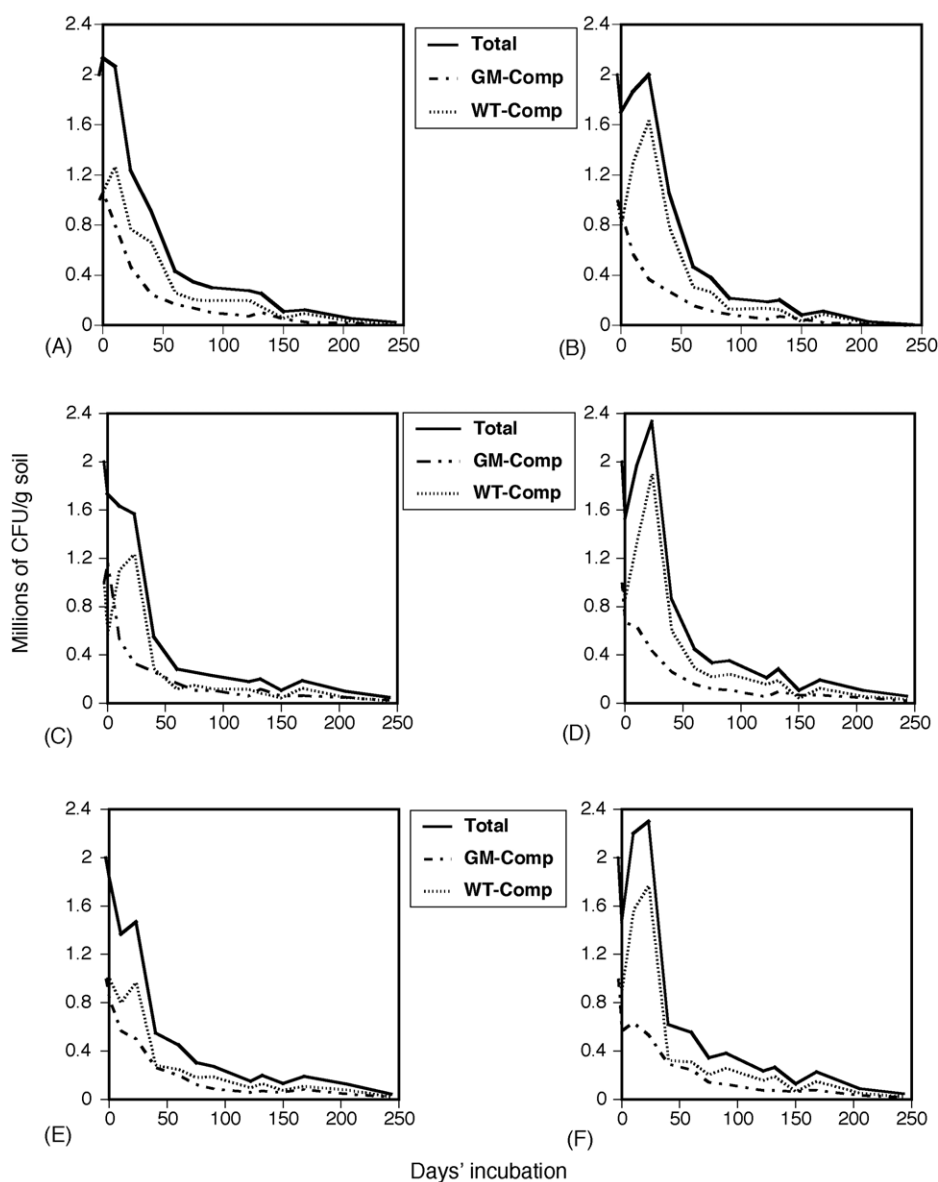


Fig. 4. Population dynamics of *T. vires* strains in competition in soil mesocosms. Wild type (WT), genetically modified (GM) and total population dynamics in incubation temperature regime I (constant 27°C) (A and B), II (slow temperature cycle) (C and D) and III (abrupt temperature cycle) (E and F). Population dynamics in soil mesocosms are presented in panels (A, C and D); dynamics in soil mesocosms amended with dried, milled cotton roots are presented in (B, D and F).

significant differences in the survival curves for the wild-type versus the genetically engineered strain ($P < 0.01$), and for the survival in amended versus non-amended soil ($P < 0.01$). Much of the variation between the survival curves was in the presence or absence and the magnitude of an increase in the

populations shortly after the initiation of the experiment.

Regression models were constructed to evaluate the effect of incubation time, amendment, replication, temperature regime and strain had on the population size of *T. vires* in the soil mesocosms. Observed

Table 1
Effect of individual variables on the *T. virens* population

| Variable | R^2 | P-value |
|-------------|-------|---------|
| Time | 0.613 | <0.001 |
| Strain | 0.054 | <0.001 |
| Temperature | 0.027 | <0.001 |
| Amendment | 0.002 | 0.172 |
| Replication | 0.002 | 0.347 |

populations were converted to natural log of population size plus 100 to normalize the error across sampling dates. The effects of each factor are presented in Table 1. A predictive model was developed that included incubation time, strain, temperature regime and amendment and accounted for 70% of the observed variance in the population size (Table 2 and Fig. 5).

3.4. Molecular estimation of population size

The population sizes that were determined through serial dilutions and plating on selective media were compared to the estimated population sizes as determined by QC-PCR. After 243 days' incubation, the population in various treatments varied from the limit of detection up to 1×10^5 CFU/g soil. Population estimates by QC-PCR ranged from 6×10^4 CFU/g soil to 8.9×10^6 CFU/g soil. Similar population dynamics were observed with both approaches yielding results that were qualitatively similar however, the absolute population size differed between the two methods by more than a log order of magnitude (Fig. 6).

Table 2
Multiple regression model of *T. virens* populations in soil

| | |
|------------------------------|----------|
| R^2 | 0.696 |
| Observations | 936 |
| Intercept | 13.71 |
| Parameter | Estimate |
| Constant temperature | −0.388 |
| Mild fluctuating temperature | 0.170 |
| CR amended soil | 0.074 |
| GM strain | −0.266 |
| GM strain in competition | −0.415 |
| WT strain in competition | 0.579 |
| Time | −0.018 |

Values expressed as thousands of CFU/g soil.

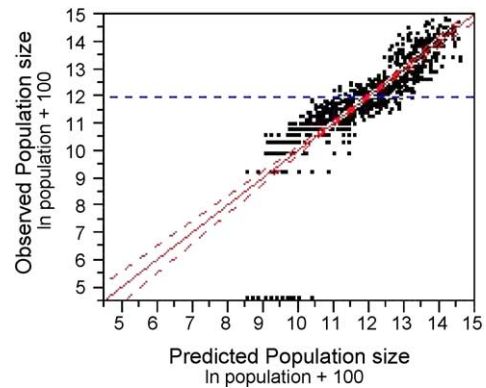


Fig. 5. Fit of observed population vs. modeled population. Dotted line shows 95% confidence interval.

3.5. Responsiveness of populations

When the fresh cotton root segments were recovered after brief incubation in the mesocosms, washed and placed directly on selective media, all segments, from all treatments were colonized. Recovered root segments were washed, homogenized and plated on selective media to characterize each strain's relative colonization efficiency. At the beginning and the end of the experiment, the two strains colonized with similar efficiency (Fig. 7A). Between days 100 and 200, the WT strain was recovered from colonized roots at a significantly higher percentage.

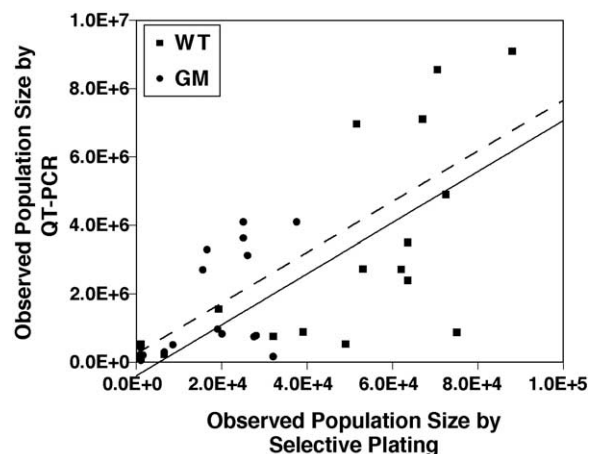


Fig. 6. Agreement between estimates of population size between selective plating and QC-PCR. Solid line for wild type (WT) population. Dashed line for genetically modified (GM) population.

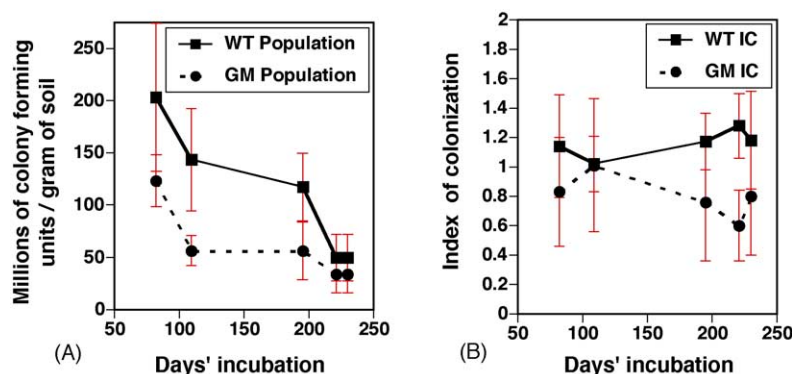


Fig. 7. Colonization of cotton root segments placed in soil mesocosms. Solid line for wild type (WT) population. Dashed line for genetically modified (GM) population. Total colonization in (A). Index of colonization (IC) in (B).

An index of colonization was developed to measure the efficiency of colonization for each strain at each time point by dividing the percent colonization for each strain by each strain's population size (Fig. 7B). After thus correcting for the smaller population size of the GM strain at the intermediate time points both strains had similar colonization efficiencies. After 243 days, the efficiency of colonization was similar to the efficiency at the beginning of the incubation.

3.6. Transgene stability

After 250 days' incubation, and in the absence of selection pressure on *hygB* (no addition of hygromycin to mesocosms), there were no observed losses of the transgene (*opd*) in the 1300 colonies examined. All colonies expressing resistance to hygromycin were also capable of producing OPH in the presence of paraoxon. Furthermore, neither hygromycin resistance nor OPH production were observed in the WT monocultures, indicating that there was any spontaneous mutation for these traits and there was no detectable contamination.

4. Discussion

T. virens is among the best-studied of biocontrol agents, with a history spanning over 60 years and documented utility on numerous host crops including fruits, vegetables, agronomic crops and turf (Howell, 1998; Weindling, 1932). The GM strain of *T. virens* in the present study has also been particularly well-

studied. This strain was one of the first stably transformed filamentous fungi (Dave et al., 1994). The population dynamics of the GM strain in the rhizosphere and the hyphal extension and rate and branching pattern has been compared to the WT strain (Classen et al., 1996, 2000; Cross and Kenerley, 2004). These comparisons between the GM and WT strain revealed significant quantitative differences, such as hyphal extension rate and the efficiency of root colonization, but, overall, similar growth patterns.

The present study is, in part, a validation of these earlier findings. The stability of the transgenes, in all temperature regimes, in monoculture and in the presence of the wild-type, parental strain for 243 days in the absence of any selection pressure strongly suggests the long-term persistence of these transgenes. The broad ecological adaptability of *T. virens* and other, related *Trichoderma* spp. (Behera et al., 1991; Bokhary and Parvez, 1995; Classen et al., 1996; Deka and Mishra, 1983; Howell, 1996; Klein and Eveligh, 1998) coupled with its tolerance to many xenobiotics underscore a need for caution before the field deployment of genetically engineered forms of these organisms. The results of the present study indicate that the GM strain is similarly persistent as the WT strain and that the transgenes were stable and expressed over the duration of the experiment.

Treatments that included the WT and GM strains together in the same soil mesocosm were initially conceived of as 'competition' experiments. In fact, there is no evidence of an interaction between the populations during co-incubation; the presence of one strain had no readily discernable effect on the

population dynamics of the other, ‘competing’ strain. Classical population models (Lotka, 1956) are most appropriate for growing populations, although other models have been developed specifically to account for populations with this general dynamic (Wu et al., 2000). The conditions of the present study only permitted a brief, initial increase in the populations, followed by a slow decline in the population size. With this early growth and the long, slow decline there may have been limited opportunity for any negative interstrain interactions. An index of colonization was developed to generally assess the responsiveness of the populations after long incubation in soil mesocosms. The ability to colonize introduced substrates rapidly, when adjusted for population size, did not meaningfully change over the course of the study. While the population size declined over time, the responsiveness of the remaining population did not diminish.

There was a noticeable difference in the population sizes of the two strains when determined by QC-PCR as compared with the results of dilution plating on selective media. During the initial validation of the QC-PCR method for *T. virens*, the immediate extraction of DNA from soil infested with conidia yielded comparable population sizes. The initial study demonstrated the QC-PCR was more sensitive than dilution plating and probably detects non-culturable cells (Baek and Kenerley, 1998). The present study demonstrated that these differences might be as great as a log order of magnitude. After 243 days’ incubation in soil, the populations studied here were almost entirely composed of chlamydospores. These propagules are multi-nucleate (Ossanna and Mischke, 1990) and freshly cultured chlamydospores have a germination rate of approximately 75%. This germination rate decreases with time (Kenerley, unpublished), possibly resulting in gene detection but not propagule counts upon dilution plating. Of further interest would be determining if the differences realized also reflect the presence of target DNA from the GEM freely associated in the soil.

5. Conclusion

There were quantitative differences between the GM and WT strains in population dynamics and

responsiveness, but the observable qualities were overwhelming similar: both strains had similar persistence over long-term soil incubation, both rapidly and efficiently colonized natural substrates and each maintained their unique genetic and phenotypic identity even in the presence of the other strain.

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